AFRL-SR-AR-TR-03-
0324

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (*Leave Blank*)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 30, 2003	Final Technical Report 1 Sep 99 to 31 Mar 03

4. TITLE AND SUBTITLE
JP8+100 Jet Fuel Toxicity: Proteomic Analysis

5. FUNDING NUMBERS
F49620-99-1-0153

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9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AFOSR/NL
801 North Randolph Street, Room 732
Arlington VA 22203-1977

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

20030915 028

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approval for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words) This final technical report describes the results of experiments that were undertaken to analyze the effect of JP8 jet fuel exposure by aerosol/vapor on quantitative and qualitative gene expression in rodent tissues. The stated objectives were to 1) generate gene expression databases for some of the major rodent target tissues, 2) identify as many of the affected gene products as possible, and 3) apply the observed molecular alterations to elucidating JP8's multifaceted toxicity. As a result of our efforts we have determined that both acute and chronic JP8 exposure significantly alters protein expression in a range of target organs, even after a period of recovery, and that these changes correspond to histological observations in those organs. Furthermore, the protein alterations observed in rats and mice are suggestive of potential hazard when extrapolated to humans.

14. SUBJECT TERMS

15. NO. OF PAGES 29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	

NSN 7540-01-280-5500
Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18 298-102

FINAL TECHNICAL REPORT

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Abstract

This final technical report describes the results of experiments that were undertaken to analyze the effect of JP8 jet fuel exposure by aerosol/vapor on quantitative and qualitative gene expression in rodent tissues. The stated objectives were to 1) generate gene expression databases for some of the major rodent target tissues, 2) identify as many of the affected gene products as possible, and 3) apply the observed molecular alterations to elucidating JP8's multifaceted toxicity. As a result of our efforts, we determined that both acute and chronic JP8 exposure significantly alters protein expression in a range of target organs, even after a period of recovery, and that these changes correspond to histological observations in those organs. Furthermore, the protein alterations observed in rats and mice are suggestive of potential hazard when extrapolated to humans.

The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, DHHS, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

1 Lung proteomics – response to various JP8 exposure levels

1.1 Introduction

Jet Propulsion-8 (JP-8) jet fuel is a complex mixture of aliphatic and aromatic hydrocarbons similar to kerosene that is the primary fuel of both NATO and US armed forces. JP-8's relatively low vapor pressure (1.8 mmHg) and high flash point (38 8C) reduces the incidence of crash-induced fires, explosions, and evaporative losses (Mattie et al., 1991). The physical and chemical attributes of JP-8 make it a desirable alternative to earlier fuels (i.e. JP-4). Unfortunately, JP-8's physical characteristics reduce vaporization and result in increased bioavailability and human exposure. Exposure to military and civilian personnel ranges from immediate association with JP-8 jet fuel service and system maintenance to those individuals who are neither on nor near any aircraft or JP-8 jet fuel-related flight-line activities. The latest personal exposure study on US Air Force Bases demonstrates that experienced fuel system maintenance personnel exhibit chronic elevated levels of JP-8 jet fuel compounds in their breath that are 40 times higher than ambient levels. Even incidental civilian exposure to JP-8 via inhalation of JP-8 vapor from worker's clothes results in elevated breath levels of benzene and other fingerprint compounds (Pleil et al., 2000). Therefore, occupational and incidental exposure to JP-8 jet fuel vapor is common. Furthermore, JP-8 is very similar to Jet-A, which is used extensively in commercial jet aircraft. Significant epidemiological study has been devoted to understanding both chronic and short-term JP-8 exposure's effect on the human body. This scientific evidence suggests that JP-8 exposure has important consequential implications for human pathology (Zeiger and Smith, 1998).

Human exposure to JP-8 jet fuel primarily occurs as a result of either dermal or pulmonary exposure to JP-8 vapor, exhaust, or aerosol. The effects of chronic JP-8 exposure in rat models have included damage to skin barrier function, skin irritation, and alteration of skin structure (Kanikkannan et al., 2002). Additionally, JP-8 jet fuel has been shown to suppress immune response in mice (Harris et al., 1997; Ullrich, 1999; Harris et al., 2000, 2001; Ramos et al., 2002). Significant alterations in brain pathology in rats have been reported for both short- and long-term chronic JP-8 exposures (Rossi et al., 2001; Ritchie et al., 2001). A reduction in the fetal body weight presents serious risk to the 11% of active-duty women Air Force personnel of child-bearing age (Cooper and Mattie, 1996). DNA damage has been detected in rat hepatoma cells in vitro (Grant et al., 2001). Chronic exposure to commercial jet fuel, similar in composition to JP-8, has even been linked to personality changes and emotional dysfunction (Struwe et al., 1983), and neurasthenia, psychasthenia, and other psychiatric symptoms (Knave et al., 1979, 1978; Mindus et al., 1978). The wide-ranging effects of JP-8 jet fuel exposure can most likely be attributed to its variable routes of delivery and its complex composition of over 2000 constituents.

Particularly relevant to this study is the extensive research of JP-8's toxicity in the pulmonary system. The development of nose-only JP-8 jet fuel simulated exposure protocols in rodents has allowed for in-depth evaluation of systemic injury due to JP-8 aerosol exposure (Hays et al., 1995). Lung epithelial cell apoptosis has been identified as a result of 1-h/day nose-only JP-8 exposure for 7, 28, and 56 day exposures at concentrations of 469-520 mg/m3 (low dose) and 814-1263 mg/m3 (high dose) (Pfaff et al., 1996). Morphological lung injury has been identified at doses of 50 mg/m3, which is well below the current inhalation safety standard of 350 mg/m3 (Robledo et al., 2000). Lung injury in the form of increased epithelial permeability (indicated by increased clearance of ^{99}technetium-labeled diethylenetriamine pentaacetate) was dose-related to JP-8 jet fuel exposure (Hays et al., 1995). Interstitial edema, alveolar septal damage, alveolar epithelial type II cell injury, and accumulation of inflammatory cells all resulted from high dose (520 mg/m3) 28-day exposure groups (Pfaff et al., 1995). These results demonstrate the histological and morphological damage caused to the pulmonary system as a direct effect of chronic JP-8 jet fuel exposure.

Even with the extensive data illustrating JP-8's damaging effects on normal respiratory structure and function, little is known regarding the molecular mechanism(s) responsible for these responses, we previously presented the first major lung protein analysis of JP-8 exposed lung tissues. Those findings indicated significant changes in protein expression. We found decreased abundance in processing and

translocation proteins, which suggest impairment of the cells protein synthesizing machinery. Additionally, ultrastructural damage to alveolar epithelial type II cells via loss of microvilli function was suggested in the decreased expression of Ulip2 (also known as unc-33-like phophoprotein and dihydropyriminidinase-related protein-2), a non-covalently associated membrane protein likely involved in signal transduction (Witzmann et al., 1999). Despite these initial studies into JP-8-related proteomics, a major gap remains in our understanding of JP-8 toxicity.

This portion of our study sought to elucidate the mechanisms of JP-8 toxicity, specifically relating to lung cell apoptosis and edema. Pulmonary exposure to aerosolized JP-8 jet fuel resulted in significant cylosolic protein expression alteration. At 1000 and 2500 mg/m3 jet fuel exposure, a total of 120 and 165 proteins were altered, respectively. Furthermore, a 250 mg/m3 JP-8 jet fuel exposure level resulted in the alteration of 41 proteins. Several of these identified proteins are related to apoptosis, protein repair, and cell structure indicating significant cell response to JP-8-related exposure.

1.2. Methods

1.2.1. Reagents

Sequence-grade, modified trypsin was obtained from Promega (Madison, WI). Ultrapure electrophoretic reagents were obtained from Bio-Rad (Richmond, CA), Sigma (St. Louis, MO) BDH (Poole, UK) and National Diagnostics (Atlanta, GA). CHAPS (3-[(3-cholamidopropy1)-dimethylammonio]-1-propanesulfonate) was obtained from Calbiochem (La Jolla, CA) and iodoacetamide from Sigma. All other chemicals were reagent grade.

1.2.2. Animals and JP-8 exposure

Male Swiss-Webster mice (18-20 g) were used in this study. Thirty mice were randomly assigned into two groups, N=15/group, for either JP-8 jet fuel exposure or controls. Briefly, as in our previous study (Robledo and Witten, 1998), JP-8 jet fuel blend (obtained from Wright-Patterson AFB Fuel Laboratory, OH) aerosol was generated using an Ultra-Neb Model #99 nebulizer (DeVilbiss, Somerset, PA). The aerosolized JP-8 vapor was allowed to mix with ambient air after which it was drawn through a 24-port IN-TOX nose-only inhalation chamber using a constant vacuum flow of 0.143 l/min, Total daily exposure time was 1 h repeated for a total of 7 days. This equated to an average JP-8 jet Fuel exposure concentration of 250, 1000 and 2300 mg/m3. JP-8 jet fuel concentrations and particle sizes were determined using a seven-stage cascade impactor (IN-TOX). Control animals were handled in an identical manner to the JP-8 group except that they were exposed to ambient air. The IN-TOX chamber is designed for nose-only exposure to minimize oral ingestion of JP-8 jet fuel during grooming, which more accurately simulated occupational exposures. Euthanasia of the mice occurred on day 7 via CO_2 asphyxiation.

1.2.3. Sample preparation

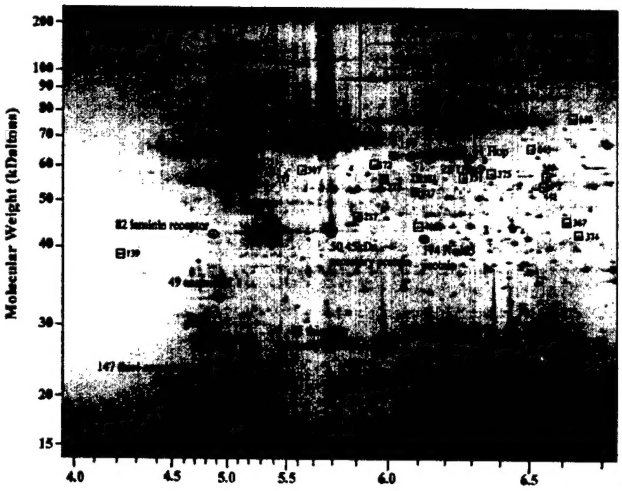
Lung tissue was homogenized in five volumes of ice-cold 20 mM Tris□/HCl, pH 7.8, 2 mM EGTA, 10 mM EDTA, 2 mM dithiothreitol (DTT). Two different sample forms were prepared based solely on their being prepared during separate experiments. For 1000 and 2500 mg/m3 exposure levels, lung cytosols were prepared by centrifugation at 100 000 x g for 20 min and solubilized in one volume of lysis buffer (pH 9.5) containing 9 M urea, 4% CHAPS, 1% DTT and 2% carrier ampholytes (pH 8-10.5). For the 250 mg/m^3 exposure level, whole lung tissue samples were prepared in a similar manner.

1.2.4. Two-dimensional electrophoresis

Sample proteins were resolved by 2-DE using a 20□/25 cm 2-DE gel system (20 gels per run) (Anderson, 1991), 175 mg were applied to each isoelectric focusing (IEF) gel tube; gels were run for 25 000 V h using a progressively increasing voltage protocol. A computer-controlled gradient casting system was used to prepare second-dimensional SDS gradient slab gels (11□/17% T). First-dimensional IEF tube gels were loaded directly onto the slab gels without equilibration. Second-dimensional slab gels were run at 8°C for 18 h at 160 V and then stained for 96 h using a colloidal Coomassie Blue G-250 procedure (Neuhoff et al., 1988) (Figs. 1 and 2 on the next page).

1.2.5. Gel pattern analysis

Stained gels were optically scanned at 121 um resolution using a CCD scanner, and images were



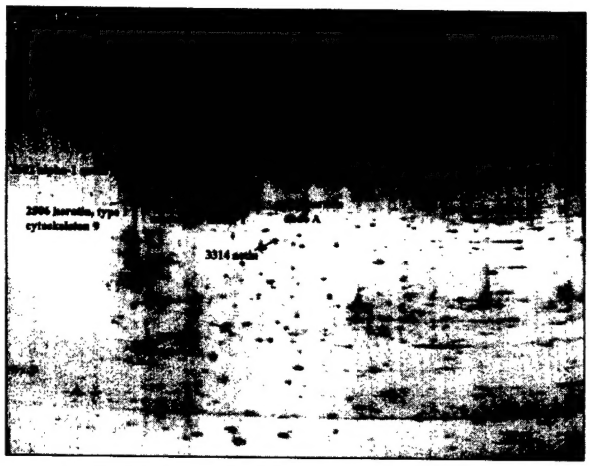


Fig. 1 (left). Coomassie blue-stained 2-DE gel pattern of Swiss-Webster mouse lung cytosol. Numbered spots in squares (I) are proteins that were altered by JP-8 jet fuel exposure while circled (k) and labeled spots are proteins that were tryptically digested, yielding masses subjected to MALDI-MS for submission to a peptide mass database for protein identification. Molecular weight an pI calibrations are estimates based on the calculate MW and pI of protein spots identified in the pattern.

Fig. 2 (right). Coomassie blue-stained 2-DE gel of Swiss-Webster mouse lung. Labeled proteins were identified by mass via a peptide mass database. Numbers associated with the proteins are their respective SSP number.

processed as described (Anderson et al., 1994). Groupwise statistical comparisons were made to screen for protein alterations (Student's t -test).

2.6. Peptide mass fingerprinting

Protein spots whose abundance differed between exposure groups along with other spots from the stained and image-analyzed two-dimensional gels were cut from the gel robotically using the Protean™ 2D Spot Cutter (Bio-Rad), placed in each of 94 wells of a 96-well plate, along with one gel blank and scrum albumin in acrylamide, and processed using the MassPREP Station (MicroMass). In this automated system, the excised protein spots were destained with 100 mM ammonium bicarbonate-50% acetonitrile followed by 100% acetonitrile, reduced with 10 mM DTT in 100 mM ammonium bicarbonate, alkylated with 55 mM iodoacctamide in 100 mM ammonium bicarbonate, and tryptically digested using Promega sequence-grade, modified trypsin at a final concentration of 13 ng/ml in 100 mM ammonium bicarbonate for 14 h (overnight). The resulting peptides were extracted by addition of 10 ml trypsin solution to the wells and refrigerating the plate for 30 min. Four microliter of peptide extract was then placed onto each corresponding position (A1-H12) on the MALDl target plate, air dried, and the application repeated until all extract buffer was used up. When the peptide sample targets were dry, each was overlayed with 1 ml of matrix (10 mg/ml a-cyano-4-hydroxycinnamic acid - 0.05% trifluoroacetic acid) and analyzed by MALDI-TOF-MS using a MicroMass M@LDI System (MicroMass). Prior to data collection, the instrument was calibrated using peptide standards and internal standards based on tryptic autolysis peaks (842.5099 and 2211.1045 Da) were used for calibration. Proteins from the 250 mg/m3 exposure group were identified by manual Profound™ (Proteometrics LLC) database searches using the mass lists obtained from MALDI spectra of 188 spots cut from the gels. A Z-score of 1.30, corresponding to the 90th percentile, was the threshold for what was considered a positive identification. Individual peptides from the 100 and 2500 mg/m3 exposure group were not identified as a result of the peptide identifications not being matched to the higher dose patterns. Identification of peptides from those groups would have been speculative.

1.3. Results

Pulmonary exposure to aerosolized JP-8 jet fuel resulted in significant (PB/0.05) cytosolic and whole lung protein expression alteration, At 1000 mg/m^3 jet fuel exposure, a total of 120 proteins

were altered in the lung cytosol tissue. Specifically, 21 proteins exhibited increased abundance and 99 showed decreased abundance. At 2500 mg/m3 jet fuel exposure, a total of 165 proteins were altered in the lung cytosol tissue. Of these, 30 exhibited increased abundance while 135 demonstrated decreased abundance. Furthermore, a 250 mg/m3 JP-8 jet fuel exposure level resulted in the alteration of 41 proteins in whole lung samples. Thirty-one of those proteins exhibited increased abundance while 10 showed decreased abundance (Table 1). The identified proteins are presented in Table 2 with average abundance of each group, the change in the abundance of treated samples relative to the untreated, and the probability as calculated by Student's t -test.

Table 1
Proteins altered by aerosolized JP-8 jet fuel exposure in mice lung ($P < 0.05$)

Tissue	Exposure (mg/m^3)	# Altered	Increased	Decreased
Whole lung	250	41	31	10
Lung cytosol	1000	120	21	99
Lung cytosol	2500	165	30	135

Of the altered proteins, seven identified proteins were found to be significant markers of JP-8-induced stress on lung epithelial cells. Specifically, decreased expression of a1-anti-trypsin (AAT) from 2869.6 for controls to 2296.2 for JP-8 exposed subjects has potential implications for the development of chronic obstructive pulmonary disorder (COPD) and pulmonary emphysema. Furthermore, four other proteins related to cell structure, including actin, keratin type 1 cytoskeleton 9, desmin fragments, and cytokeratin endo A (CKA), were upregulated. Increases in protein expression were also observed for heat shock protein 60 (HSP60), which is related to protein mis-folding repair, and Fas-associated factor 1 (FAF1), whose role involves mediating cell apoptosis. Alteration of all seven protein's expression correlates with histological data demonstrating JP-8 jet fuel puts significant stress on lung epithelium and pulmonary function.

1.4. Discussion

1.4.1. Cellular ultrastructure-associated protein upregulation

Proteomic expression alterations in this study correlate with previously identified histological analysis of pulmonary interstitial edema resulting from endothelial damage. Four intermediate filament proteins (keratin type 1 cytoskeleton 9, actin, desmin fragments, and CKA) required for maintaining cell structure and/or differentiation were identified as exhibiting unregulated expression following JP-8 exposure. These data demonstrate that the expected pulmonary protein response, i.e. increased expression of cell structural and differentiation proteins, correlates with the pathological response of JP-8-induced interstitial edema (Hays et al., 1995). All intermediate filament proteins, including those relevant to this study, (keratin type 1 cytoskeleton 9, actin, desmin fragments, and CKA) are capable of forming intermediate filaments as a result of their a-helical domains. Specifically, keratin type 1 cytoskeleton 9 is a type I acid keratin, which includes K9-K20. This group's interactions with basic-neutral type II keratins (K1□/K8) form distinct combinations that are specific to the type of epithelial cell, which allows for different intermediate filament function (Hutton et al., 1998; Galou et al., 1997). Keratins, such as K9 and CKA, are the hallmarks of epithelial differentiation and proliferation (Schlage et al., 1998). As early studies of the regrowth of epithelium establish, keratins migrate to the sight of injury and proliferate as a result of the epithelial injury (Paladini et al., 1996). Therefore, an increase in lung epithelial K9 and CKA are expected in light of the JP-8-induced pulmonary edema and epithelial apoptosis (Hays et al., 1995), indicating lung epithelial hyperplasia and a cell-mediated response to improve cell integrity at the injury site. This expected increase was confirmed by the experimental data.

Table 2
250 mg/m^3 JP-8 jet fuel aerosol-mediated protein expression alterations in identified proteins

SSP	Identity	Protein abundance		
		Control	JP-8	Prob
3314	**Actin**	**1038.3**	**1546.7**	**0.001**
p414	Actin, alpha	5348.4	5987.3	0.5
4404	Actin-related protein	400.6	491.5	0.4
6516	Aldehyde dehydrogenase	4277.9	4558.7	0.4
8311	Aldose reductase	1532.6	1708.8	0.6
1602	**AAT 1–4**	**2869.6**	**2296.2**	**0.02**
4304	Annexin III	2057.7	2414.2	0.06
1307	Annexin V	4054.4	5011.8	0.4
6107	Antioxidant protein 2	7325.2	7231.8	0.9
3102	Apolipoprotein A-I	5235.0	4492.5	0.2
2501	ATP synthase, beta chain	2926.3	3275.9	0.2
5820	Brefeldin A-inhibited guanine nucleotide-exchange protein	140.1	161.9	0.5
712	Calreticulin	1710.9	1835.8	0.6
8409	Creatine kinase	2203.6	1889.5	0.6
4517	**CKA**	**1007.9**	**1365.2**	**0.03**
3501	**Desmin fragment**	**102.3**	**137.4**	**0.03**
6612	Dihydropyrimidinase related protein-2	1278.0	1681.3	0.04
7502	Enolase, alpha	3325.3	3117.5	0.8
6511	Enolase, alpha (charge variant)	1274.6	1387.0	0.4
5604	ER60	1534.8	1754.9	0.1
5720	Ezrin	991.7	1149.5	0.2
2523	**FAF1**	**349.9**	**503.6**	**0.05**
2701	grp78	2755.6	3009.0	0.1
1802	grp94 (endoplasmin)	1931.8	2513.2	0.3
8708	Hexokinase, type 1	1131.6	714.0	0.05
3706	hsc70	3634.9	3919.1	0.4
3607	**HSP60**	**1295.2**	**1753.1**	**0.007**
p506	**Keratin, type 1 cytoskeletal 9**	**190.4**	**266.7**	**0.02**
7707	Moesin	1233.5	1265.6	0.9
7715	Moesin	873.8	964.0	0.6
2105	Myosin light chain 1	385.6	108.7	0.1
3609	P21 activated kinase 1B	198.5	278.5	0.0004
4217	PA28, alpha subunit	950.8	1173.3	0.06
5513	Selenium binding protein 1	2335.5	2698.8	0.05
5503	Selenium binding protein 2	1346.0	1528.2	0.3
6002	Superoxide dismutase	3944.0	4635.8	0.1
5204	Thioether *S*-methyltransferase	5806.5	6883.5	0.08
3901	Ubiquitin carboxyl-terminal hydrolase 2	3048.0	2818.7	0.6
2611	Vimentin	1444.2	1217.4	0.3
5817	Vinculin	3413.0	3185.2	0,5
5811	Vinculin (charge variant)	571.6	632.3	0.4

Similarly, the actin cytoskeleton is critical to the intermediate filament support function within the cell, and serves as a critical structure by which recruited keratin proteins can migrate to epidermal wounds (Martin, 1997). Therefore, the observed increase in actin microfilaments correlates with keratin relocation to the JP-8-induced injury site. Additionally, desmin fragments, while not structural proteins, are muscle-specific intermediate filament proteins that are early markers of muscle precursor cells (Kaufman and Foster, 1988; Kaufman et al., 1991; Yablonka-Reuveni et al., 1999). During muscle injury, myoblasts, which are muscle precursor cells, proliferate and fuse to form myotubes that subsequently become new myofibers. Recent studies suggest that the desmin has a significant role in the structural organization and regeneration of myofibers during myogenesis (Smythe et al., 2001; Capetanaki et al., 1997; Li et al., 1994). The increased desmin expression found in this study suggests that their increased levels are related to smooth muscle regeneration following the lung epithelial edema and apoptosis.

1.4.3. Apoptosis-related protein upregulation

Extensive study has been devoted to understanding apoptotic-associated protein factors integral to selective cell death. FAF1 is a member of that cascade and has been found to enhance, yet not initiate, Fas-mediated apoptosis (Chu et al., 1995; Frohlich et al., 1998). The FAF1 upregulation in JP-8 jet fuel-exposed lungs found in this study suggests this as a component of a potential pathway leading to the previously identified JP-8- induced lung epithelial cell apoptosis (Pfaff et al., 1996) and epithelial edema (Robledo et al., 2000; Pfaff et al., 1995).

FAF1 has two ubiquitin conjugating enzymelike domains, a chromatin assembly factor-like domain, and a unique nuclear localization signal unlike the classical motif (Ryu et al., 1999). However, the exact nature of the FAF1's interaction in the Fas-mediated apoptosis remains largely unknown. While two distinct Fas-associated apoptosis pathways have been postulated (Chang et al., 1998; Medema et al., 1997), it does appear certain that Fas and FAF1 are related solely to apoptosis (Ryu and Kim, 2001). The over-expression of FAF1 in this study suggests this as a key mechanism in the Fas-mediated apoptosis in JP-8-exposed lung epithelial cells. Such a finding is also consistent with the extensive JP-8-induced lung epithelial edema (Pfaff et al., 1995), and with this study's proteomic evidence suggesting lung epithelial hyperplasia is occurring to counter cell apoptosis.

1.4.4. Cell 'stress' protein markers

The increased abundance of HSP60 in this study demonstrates a cell-initiated mechanism to counter the physiological stress induced by JP-8 jet fuel exposure. HSP60 is localized in the muscle mitochondria, and is thought to have a significant role in the folding and assembly of polypeptides imported into the mitochondrial inner matrix (Kaetsu et al., 2001; Wong and Wispe, 1997). Stress on the cell induces protein damage and misfolded protein structures that often leads to the epithelial cell death observed in previous studies (Pfaff et al., 1996). HSP60's role therefore, is to correct the misfolded proteins, and enable the cell to withstand increased cellular stress (Wischmeyer, 2002). The increased abundance of HSP60 in JP-8 exposed lungs found in this study demonstrates that cell-initiated mechanism to withstand the stress induced by jet fuel. This finding correlates with earlier studies demonstrating the impairment of cellular protein synthesizing machinery (Witzmann et al., 1999). Furthermore, this finding coincides with the Fas-mediated apoptosis protein upregulation demonstrated in this study, despite a significant cellular response to injury and stress in the form of HSP regulation, the lung epithelium is unable to withstand the stress that JP-8 jet fuel induces, and therefore, must initiate cell death due to the overwhelming amount of protein misfolding.

1.5. Conclusion

JP-8 jet fuel-induced lung injury presents a health risk to civilian and military personnel through incidental or occupational exposure. While extensive study has demonstrated the pathological effects of JP-8 exposure on lung epithelial tissue, little study has been devoted to analyzing JP-8 jet fuel's effects on protein expression. This study identified the upregulation of several proteins relating to cell structure, maintenance, and cell proliferation demonstrating that JP-8 exposure puts significant stress on lung epithelial cells. This proteomic response was expected in light of the previously identified lung epithelial cell edema, apoptosis, and subsequent regeneration following JP-8 exposure. Increased levels of 'stress'-related HSP's adds to evidence demonstrating the stress JP-8 jet fuel exposure has on the pulmonary system, while elucidating one of the cellular proteomic mechanisms by which the cell copes with that stress. The data correlates to previous proteomic analysis demonstrating JP-8-induced impairment of lung protein machinery. Fas-associated protein upregulation reveals a component of the cell-mediated apoptosis mechanism that was observed in JP-8-exposed lung epithelial pathology.

2 Liver & kidney proteomics - persisting alterations repeated exposure of rats to JP8 vapor.

2.1 Introduction

The goal of this portion of the study was to apply similar proteomic analysis methods, as above, to examine protein expression and protein charge modification in kidney and liver tissues from rats exposed subchronically to a target concentration of 1000 mg/m^3 JP-8 vapor, in anticipation of possible measures or biomarkers of toxicity more reliable than those utilized in previously discussed studies. This exposure, while higher than the permissible exposure limit (PEL) of mg/m^3, is equivalent to the 15-min short-term exposure limit (STEL) for JP-8 inhalation exposures recently suggested by the National Research Council, Committee on Toxicology.

2.2 Materials and methods

All methods correspond to Section 1.2 described earlier and will not be repeated. Any methods unique to this particlular phase of the study are listed below.

2.2.1 Animals and Exposures

Forty-eight (48) male Sprague-Dawley rats [Crl:CD (BR)] (50 days of age upon receipt) were procured from Charles River Breeding Laboratories, Raleigh, NC. Rats were pair housed in hanging polycarbonate shoe box cages with cellulose fiber contact bedding (Cell-Sorb Plus, A. W. Products, Inc., New Philadelphia, OH). Fresh pelleted food (Purina Formulab #5002, Purina Mills, Inc., St. Louis, MO) and fresh conditioned (reverse osmosis) water were available ad libitum, except during inhalation exposures. rats were randomly assigned to one of the following room air control or fuel vapor exposure conditions: room air control or JP-8 vapor at 1000 mg/m3 ± 10% at a flow rate of 72 L/min + 3 L/min. The whole body inhalation exposure system consisted of a fuel vapor generator, a 270-L Hinners-type room air control chamber, two fuel vapor exposure chambers identical to the control chamber, and an infrared (IR) spectrometer vapor concentration analyzer, and was similar to those used in a previous jet fuel toxicity study. The exposure chambers were filled with JP-8 vapor, or room air on a continuous basis for 6 hr/day, five days per week (Mon-Fri), for six consecutive weeks (184 hr of exposure). The control and experimental animals were exposed in one of two 8-rat stainless steel, wire mesh cages [approximately 17 cm (l) x 12 cm (w) x 17 cm (h)] within each chamber. Housing position of rats within the exposure chambers was randomized on a daily basis to minimize possible differences in regional vapor concentration. Following the completion of whole body inhalation exposures, all subjects were rested for 60 days. During this period, vapor exposed and air control rats were separately housed (conditions as described previously, except now singly housed) in air mass displacement units to minimize off-gassing effects and consequent vapor exposure of controls from the fur of exposed animals.

2.3 Results & Discussion

2.3.1 Quantitative alterations in identified proteins

Figures 3 and 4 illustrate maps of the 2-DE protein patterns of rat liver and kidney, respectively. An average of 1064 proteins were matched in the liver pattern while 895 were matched in the kidney pattern. Image analysis and statistical testing revealed significant ($P<0.001$) abundance alterations in 3 liver proteins and 4 kidney proteins following 42 days of jet fuel vapor exposure and 82 days of recovery when compared to the control group. These proteins were cut from replicate gels and subjected to tryptic digestion. Of the 7 proteins analyzed by peptide mass fingerprinting, 5 yielded searchable masses and resulted in the identification of hepatic lamin A (#L83 and L603), and renal 10-formyltetrahydrofolate dehydrogenase (#K223 and K489) and renal glutathione S-transferase (GST) (#K515) presented in Table 3. Though tryptic digestion of spot #K390 resulted in poorly resolved peptide masses, it was tentatively identified based on its homologous position, via gel matching, to rat kidney glutathione-S-transferase in our previous experiments. Spot #L601, whose abundance increased by 94%, yielded no searchable masses and remains unidentified. However, based on its coordinate position compared to #K515 in Figure 3, #L601 is resolved in a manner nearly identical to kidney GST spot #K515 and its induction would be consistent with such identity.

Table 3. Summary of MALDI-MS data obtained from Sprague-Dawley liver and kidney protein spots[a] altered after recovery from exposure to JP-8 jet fuel vapor

pI	MALDI masses matched[b]	rms Δ (Da)[c]	Peptide sequence [modification]	Protein identification (NCBI accession no.)	% Change[d]
6.54	1068.4	+0.157	(K)ELEKTYSA(K)	lamin A, , *Rattus norvegicus*, (1346413)	-50%
	1302.6	+0.043	(R)TQSSQNCSIM [farnesyl]		
	1679.7	+0.008	GSHCSSSGDPAEYNL(R)		
6.54	1068.4	+0.157	(K)ELEKTYSA(K)	lamin A, , *Rattus norvegicus*, (1346413)	+575%
	1114.5	-0.050	(R)TQSSQNCSIM [1Met-ox]		
	1302.6	+0.043	(R)TQSSQNCSIM [farnesyl]		
	1679.7	+0.008	GSHCSSSGDPAEYNL(R)		
5.79	1363.5	-0.211	(K)DLGEAALNEYLR(I)	10-formyltetrahydrofolate dehydrogenase, *Rattus norvegicus*, (1346044)	+315%
	1625.5	-0.275	(R)DTNHGPQNHEAHLR(K)		
5.79	1017.6	-0.029	(R)EFIQLLVR(K)	10-formyltetrahydrofolate dehydrogenase, *Rattus norvegicus*, (1346044)	-36%
	1264.6	+0.003	(R)FADGDVDAVLSR(A)		
	1363.6	-0.086	(K)DLGEAALNEYLR(I)		
	1540.8	-0.006	(R)ANATEFGLASGVFTR(D)		
	1552.8	-0.041	(K)GVVNILPGSGSLVGQR(L)		
	1625.6	-0.153	(R)DTNHGPQNHEAHLR(K)		
6.91	-	-	-	*tentative*: glutathione-S-transferase homolog, Mus musculus, (2393724)	+35%
6.91	955.5	+0.032	(K)ECLAHTPK(L)	glutathione-S-transferase homolog, Mus musculus, (2393724)	+57%
	994.2	-0.288	(K)LFPDDPYK(K)		
	1079.7	+0.079	(R)HEVININLK(N)		
	1361.9	+0.182	(K)GSAPPGPVPEGQIR(V)		

a) L for liver, K for kidney; spot # L601 from Figure 1 was digested but yielded no peptide masses suitable for database submission
b) matrix-assisted laser desorption mass spectrometry (MALDI) masses matched to the MS-FIT database
c) average difference between observed and calculated monoisotopic mass
d) % change in abundance relative to average abundance for each protein in the control group

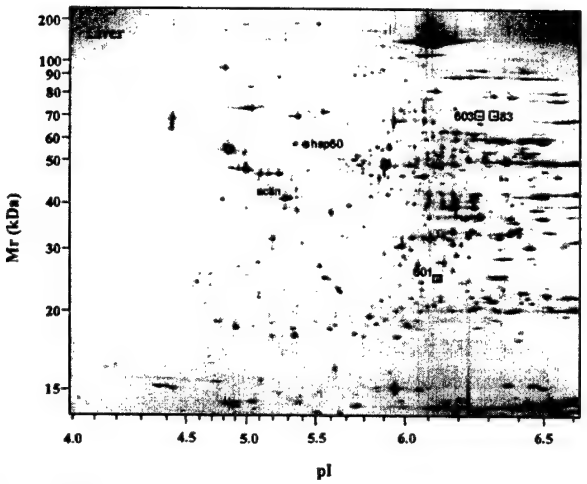
Table 4. Changes in total protein abundance and protein charge (CMI) after recovery from JP-8 jet fuel exposure

Spot #s	Protein	Control		JP-8	
		Total Abundance[a]	CMI	Total Abundance	CMI
L83 and L603	lamin A	10,736 ± 1,578	-0.09 ± 0.01	11,434 ± 1,272	-0.58 ± 0.01[c]
K223 and K489	10-formyltetrahydrofolate dehydrogenase	2,779 ± 455	-0.87 ± 0.04	3,131 ± 149	-0.53 ± 0.01[c]
K390 and K515	glutathione S-transferase homolog	5,356 ± 252	-0.42 ± 0.01	7,580 ± 1,583[b]	-0.56 ± 0.12[b]

a) Total abundance reflects sum of all charge variants; values are means ± SEM
b) $P<0.05$, control *vs.* JP-8
$P<0.001$, control *vs.* JP-8

2.3.2 Hepatic lamin A

Two proteins were independently identified as lamin A, #L83 and #L603. L83 abundance was significantly ($P<0.001$) decreased (-50%) by treatment conditions while #L603 increased by 575%. However, total abundance of lamin A (L83 + L603, Table 4) was unaffected by JP-8 exposure and recovery (10,763+1,578 vs. 11,434+1,272, control vs. 82 days post-exposure). As Figure 3 indicates, lamin A is resolved as two charge variants, L83, the most abundant in controls, and a single microheterogeneity, L603. Following exposure and recovery, there is a shift in charge from the main form (L603), which declines in abundance, to the charge variant (L83), which increases in abundance by nearly 6-fold. These individual abundance changes in lamin A demonstrate a significant ($P<0.01$) and



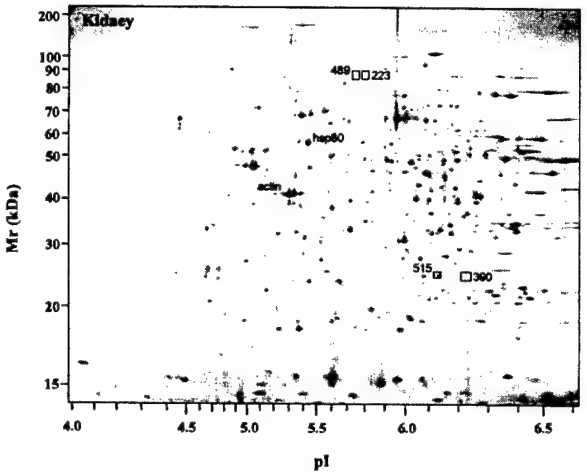


Figure 3. Coomassie blue-stained 2-DE gel pattern of male Sprague-Dawley rat liver homogenate **AND** Figure 4. Coomassie blue-stained 2-DE gel pattern of male Sprague-Dawley rat kidney homogenate.
Numbered spots in squares (□) are proteins that were altered by JP-8 jet fuel exposure and tryptically digested yielding masses subjected to MALDI-MS for protein identification. All but one (#601) of the circled spots were identified. Molecular weight and pI calibrations are estimates based on previously calibrated gel patterns of similar tissues run under virtually identical conditions. Actin (β and γ) and hsp60 are identified in this figure simply for the reader's reference and orientation. Their identities are based on their coordinate positions homologous to previously identified proteins from samples resolved under similar running conditions in this laboratory and others.

persisting modulation in the protein charge modification index (CMI) between groups and suggest a considerable post-translational modification not observed in controls. While the nature of this modification provisionally remains unknown and the functional significance of this finding remains conjectural, it would appear probable that this result indicates either long-term, post-exposure hepatic disease and/or evidence of significant and continued hepatic regeneration from JP-8-mediated consequences.

The nuclear lamina of hepatocytes and many other cell types, a meshwork of intermediate filaments closely apposed to the inner nuclear membrane, is believed to provide a framework important for nuclear envelope integrity and interphase chromatin organization. Its assembly state is regulated in the cytoplasm (Kitten & Nigg, 1991) by phosphorylation of its protein components, minimally including lamins A, B, and C. Isoprenylation of the lamins is essential for their proper membrane anchoring and functionality. Normal and regenerating hepatocytes, biliary epithelial cells (ductal and ductular cells), and hepatocellular carcinoma cells invariably expressed both A-type lamins and lamin B (Hytiroglou, et al., 1993). Enhanced prenylation of proteins is observed during rat liver regeneration (Bruscalupi et al., 1997) and may be either an additional or alternative explanation for our observations.

2.3.3 Glutathione-S-Transferase

Kidney protein spots #K390 and #K515 were identified as GST homologs. According to our own previous results, the coordinate position of these proteins matches those identified as GSTP1 (π class). The GSTs are a multi-gene family of enzymes responsible for the detoxification or, infrequently, the activation of a wide range of xenobiotics (Eaton & Bammler, 1999). The cytosolic family of GST consists of several families (α, μ, π, δ and θ) with catalytically active GST formed as homo- and heterodimers from subunits within each family. GST substrates include environmental carcinogens, environmental toxicants (e.g., acrolein, methylene chloride), pesticides (e.g., lindane, DDT, methyl parathion), pharmaceuticals (e.g., acetaminophen, nitroglycerine), and endogenous molecules (endogenous fatty acid oxidation products, prostaglandin A2, prostacyclin 2, and catecholamine reactive species). GST catalyzes the nucleophilic activity of glutathione on electrophilic substrates, decreasing their toxic effects on cellular macromolecules (Armstrong, 1997). Therefore, although the physiological significance of GSTs is largely unknown, their high catalytic activity toward a wide diversity of endogenous and exogenous reactive species is thought to be protective against oxidative tissue damage.

Like lamin A, GST is resolved as two charge variants whose abundance is altered by JP-8 exposure and recovery (Table 3) as is total GST homolog (sum of #K390 and #K515 abundances, Table 2). CMI

calculation (Table 4) confirmed the chemical modification of GST homolog, a 33% increase in CMI. Though the nature of the charge shift has not yet been elucidated, it may be due to either increased deamidation of glutamine or asparagine residues (Funakoshi & Deutsch, 1968), or the phosphorylation of serine, thyrosine, or threonine residues (Bulavin et al., 1996). Because GST elevation is frequently observed and interpreted as indicative of toxic exposure or effect these data suggest that GST is indeed a good marker of jet fuel intoxication, even 82 days after the last exposure.

2.3.4 10-Formyltetrahydrofolate Dehydrogenase (10-FTHF DH)

Two additional kidney proteins were identified independently as 10-FTHF DH, resolved as charge variants #K223 and #K489 in Figure 4. As Table 3 indicates, the main, unmodified #K223 underwent a 315% increase in abundance with exposure and recovery while the more acidic #K489 was decreased by 36%. This rightward charge shift is contrary to our observations regarding lamin A and GST, where chemical modification calculated as CMI was increased. As Table 4 indicates, total 10-FTHF DH total abundance was unaltered but a significant ($P<0.001$) decline in charge modification resulted after exposure and recovery. Cytosolic 10-FTHF DH catalyzes two reactions: the NADP+-dependent oxidation of 10-formyltetrahydrofolate to tetrahydrofolate and CO2 and the NADP+-independent hydrolysis of 10-formyltetrahydrofolate to tetrahydrofolate and formic acid (Cook & Wagner, 1995). 10-FTHF DH exists as a tetramer of 99 kDa subunits in rat liver and contains a conserved active site cysteine (Cys-707). The modification observed in control 10-FTHF DH as charge variant #K489 and a CMI of –0.87 may be due to in vivo covalent binding of tetrahydrofolate polyglutamates (Wagner et al., 1995). However, the explanation for the decline in the abundance of the modified charge variant and the reduction in CMI to –0.53 is unsettled. It may be a reduction in the number of covalently bound folate polyglutamates, formation of phosphorates, addition of chemical adducts or polymorphism. Furthermore, the effect this difference has on enzyme activity in this study is unknown. Although not identified in the liver pattern, protein spots in coordinate positions homologous to kidney 10FTHDHNonetheless, it is clear that 6 weeks of JP-8 vapor exposure followed by 82 days of recovery has significant effects on the expression of 3 important proteins in the liver and kidney.

2.4 Conclusion

Proteomic analysis revealed quantitative and qualitative alterations in the expression of renal GST, a prominent and ubiquitous detoxification enzyme and marker of toxic exposure as well as qualitative alterations in renal 10-formyltetrahydrofolate dehydrogenase and hepatic lamin A following 42 days of JP-8 jet fuel vapor exposure in rats and 82 days of recovery. While the nature of these changes in protein expression remains to be explained by further research, these results indicate significant changes in a very narrow range of proteins after long recovery from exposure to jet fuel and suggest similar proteomic analysis at earlier time points both during exposure and recovery.

3 Testis proteomics – reproductive alterations with exposure of rats to JP8 vapor

3.1 Introduction

With respect to reproductive toxicity, few investigations have studied the effects of jet fuel exposure on the male reproductive system. Only recently, Briggs et al. (2001) reported that repeated exposure of male Sprague-Dawley rats to JP-8 in vapor phase, at levels approximating possible occupational exposures (0-1000 mg/m^3), resulted in no observable tissue histopathology or statistically significant changes in total sperm concentration or morphology. However, a linear and dose-related reduction in mean sperm motility was observed. It appears that repeated exposure to at least some chemical components of JP-8 (i.e., those contained in JP-8 vapor) induced changes in spermatogenesis or sperm motility without concomitant changes in testis histopathology. Based on the results presented by Briggs, this portion of our study attempted to identify JP-8 mediated changes at the protein level that might both explain the motility changes and identify molecular-level changes not observable with traditional histopathology. Rats were exposed to room air control conditions (0 mg/m^3), to 250 mg/m^3 (below the 8-

hr threshold limit value [TLV] for JP-8), 500 mg/m^3 (between the TLV and 15-min short-term exposure level [STEL]), or 1000 mg/m^3 (at the interim STEL) JP-8 in vapor phase for 6 hr/d (typical work shift) for 91 consecutive days. It was assumed that the vapor phase best modeled exposures most commonly encountered by military and commercial workers with direct occupational exposure to JP-8, as well those possibly encountered by the general public living near military bases, airports, or JP-8 manufacturing or storage facilities. Further, it was assumed that molecular markers of vapor exposure in rats that may occur in the absence of easily observable health consequences might prove useful in predicting human health effects from career-long exposures.

3.2 Methods

As before, only methods unique to this part of the investigation are recounted here. All other analytical methods and materials used are as described in Section 1.2.

3.2.1 Animals and Exposures

Whole body inhalation exposures occurred 6 h/d for 91 consecutive d (546 h) in four identical 670-L THRU chambers. For the daily exposures, rats were placed in individual housing spaces within one of two 8-rat wire mesh cages, within one of four consistently assigned exposure chambers. A semi-randomization procedure was used to determine daily animal placement, such that individual rats experienced vapor exposure an approximately equal number of times in each of the 16 housing locations within the assigned exposure chamber. For each vapor exposure, neat JP-8 fuel was directed through a heated J-tube, temperature controlled to maximize vaporization with minimal formation of aerosol. The vaporized fuel was introduced into a (counter-current) room air source flowing into each 670-L chamber at 55 ± 3 L/min. For control exposures, rats were identically exposed to filtered room air inflowing into an identical 670-L chamber at a rate of 55 ± 3 L/min. Tubing directing the JP-8 vapor flow was externally heated to minimize condensation, and contained a J-tube trap to contain and measure (approximately 2%) condensed vapor. Gelman 25 mm, extra thick glass fiber filters were used to sample chamber atmospheres for aerosol. Chamber atmospheres were quantified by infrared (IR) spectrometry at 20 min intervals using the IR absorbance band between 3.4 and 3.5 microns, calibrated with known mass concentrations of hexane. Chamber atmospheres were, then, adjusted by controlling raw fuel flow and/or J-tube temperature to maintain target concentrations ±10%. The four exposure group (n = 5) conditions are summarized in Table 5.

Table 5. Summary of JP-8 vapor exposure and control conditions.

JP-8 Exposure Group	Vapor Exposure (mg/m^3 ± 10%)	Days of Exposure (6 h/d)	n
Control	0	91	5
Low	250	91	5
Moderate	500	91	5
High	1000	91	5

3.2.2 Tissue preparation

Within 24-48 h post-exposure, fuel vapor exposed and air control rats were sacrificed by CO2 overdose. Testes were rapidly dissected and flash frozen for subsequent proteomics assay. Each frozen testis was bisected manually into superior and inferior halves. The inferior half was returned to the -80°C freezer, and the superior half was placed in a 50 mL beaker along with 8 volumes of a solution containing 9M urea, 4% Igepal CA-630 ([octylphenoxy] polyethoxyethanol), 1% DTT and 2% carrier ampholytes (pH 8-10.5) and thoroughly minced with surgical scissors. The minced samples were then placed in 5 mL DUALL® ground-glass tissue grinders and manually homogenized. After complete solubilization at room temperature for 120 min, samples were centrifuged at 100,000 x g for 30 min using a Beckman TL-100 ultracentrifuge to remove nucleic acid and insoluble materials, and the supernates stored at -45°C.

3.3 Results & Discussion

Figure 5 below illustrates the result of 2DE separation of rat testis proteins. The specific gel pattern shown was used as the reference pattern for Matchset construction within the imaging analysis (PDQuest) and thus serves as a suitable example of the typical testis whole homogenate protein pattern. Image analysis of all 20 gel patterns indicated that a number of testis proteins (77/1320, or 5.8%) were significantly ($p<.05$) increased or decreased in abundance following exposure to JP-8 vapor.

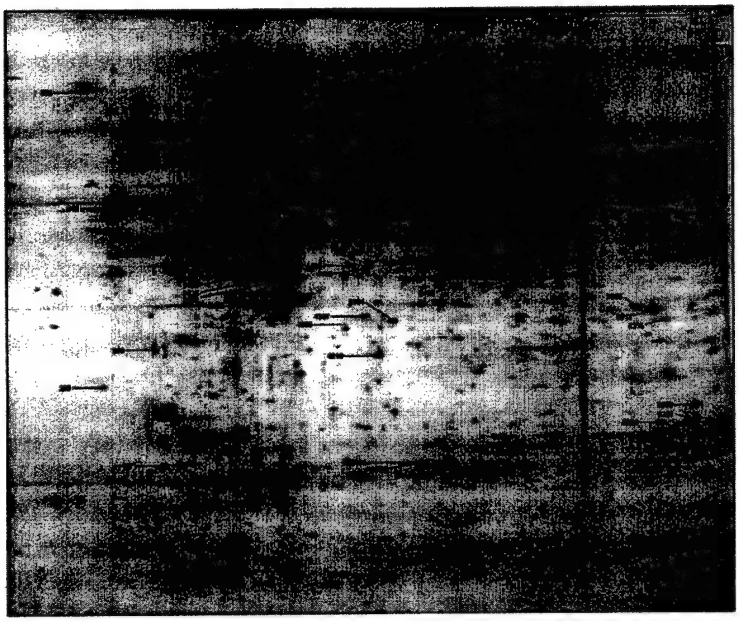


Figure 5. 2-DE pattern of rat testis proteins, solubilized and separated on 23.5 cm IEF tube gels and 20 × 25 cm slab gels, and stained with colloidal Coomassie blue as described in Section 2.6. The horizontal dimension represents a working pH gradient of approx. 4–7.5 and the vertical an acrylamide gradient of 9–19%. The protein spots were analyzed by peptide mass fingerprinting using MALDI-MS and those successfully identified are designated by their spot number (SSP) in the PDQuest reference pattern. The corresponding protein names appear in Table 6.

As shown in Table 6, repeated exposure to the lowest JP-8 vapor dose (250 mg/m^3) resulted in a 5-fold higher number of proteins with increased abundance than did exposure to the highest dose (1000 mg/m^3). In contrast, exposure to the highest dose level resulted in significantly reduced abundance of twice as many proteins as the lowest dose exposure. Across all 77 proteins altered by the various exposures, proteomic analyses of individual protein abundance, as a function of JP-8 vapor dose, seldom reflected a strict linear dose relationship. As is shown in Table 3, of the 19 identified proteins that were significantly changed in abundance at any JP-8 concentration, only 4 increased linearly in abundance as a function of dose: Hsp86, nicotinic acetylcholine receptor alpha subunit, serum albumin, and T-complex protein 1. By the same analysis, no identified protein decreased linearly in abundance as a function of JP-8 dose exposure. In the remaining 15 cases of significantly altered expression across all doses, there was no clear linear relationship between JP-8 dose (250-1000 mg/m^3) and change from baseline in protein abundance. This suggests that human risk assessment of complex mixtures like JP-8 should: 1) consider data from both low and high dose exposures; 2) not assume linear extrapolation among dose response effects; and 3) thus should not exclude non-linear dose response data. While the expression of many proteins displayed interesting trends relative JP-8 dose (some of whom appear in Table 6 listed as NC for no change), internal variation rendered these changes statistically insignificant and therefore no speculative discussion of these will be attempted.

An analysis of the functional significance of the JP-8-mediated protein expression alterations (and absence of effect) listed in Table 6 is complex, as the exact role of many of these proteins in testis function is conjectural. Additionally, many (49) of the altered proteins remain to be identified. Nonetheless, the following is an attempt to combine the observed alterations with the putative role assigned to the identified proteins altered by JP-8 vapor exposure in the literature, so that possible functional consequences in the testis can be hypothesized.

In rats exposed to 250 mg/m^3 JP-8 vapor, there was a significant two-fold increase ($p<.03$) in mitochondrial aldehyde dehydrogenase (ALDH) abundance. However, ALDH expression was unaffected in both the moderate and high exposure groups. ALDH is commonly expressed in a number of organ systems, including testis (Bedino Testore, 1998) where it is located principally in the Leydig cell and is known to increase in response to the presence of alcohols or glycols (Messiha, 1982). Furthermore, ethylene glycol monomethylether (EGME), a structural analog of DiEGME (diethylene glycol monomethyl ether), the anti-icing additive in JP-8, is metabolized by ALDH and possibly lactate

Table 6. Identified proteins from whole testis and the effect of prolonged JP-8 jet fuel exposure on mean protein abundance

SSP	z-Score	%	M_r	pI	Name	Change	Control	250 mg/m^3	p	500 mg/m^3	p	1000 mg/m^3	p
5304	1.58	22	38.7	5.7	aryl-hydrocarbon interacting protein-like 1	NC	2784	3210	0.2	2976	0.7	2789	1.0
2818	**2.22**	**24**	**111.5**	**5.1**	**150 kDa oxygen-regulated protein precursor**	**–**	**2405**	2225	0.2	**1966**	**0.05**	2158	0.6
2409	1.86	45	57.5	5.1	26S protease regulatory subunit 6B (TAT-binding protein-7) (TBP-7)	NC	947	989	0.6	1073	0.3	927	0.8
2726	2.11	40	72.1	5.1	78 kDa glucose-regulated protein precursor (grp 78)	NC	3863	3922	0.9	3631	0.6	4190	0.6
2706	**2.19**	**36**	**72.5**	**5.1**	**78 kDa glucose-regulated protein precursor (grp 78)**	**+**	**3193**	**4777**	**0.06**	3949	0.2	3620	0.5
2415	2.43	42	42.3	5.2	actin alpha	NC	3619	2063	0.3	4113	0.8	4154	0.8
3407	2.43	50	42.1	5.3	actin beta	NC	7527	8175	0.6	8407	0.6	7884	0.8
2909	**1.30**	**4**	**256.9**	**5.0**	**A-kinase anchor protein**	**+/–**	**1772**	**2547**	**0.05**	1784	1.0	1184	0.5
5416	**2.43**	**46**	**48.7**	**6.1**	**Aldehyde dehydrogenase, mitochondrial (ALDH1)**	**+/NC**	**1580**	**3330**	**0.03**	1771	0.8	1743	0.8
5507	2.43	31	48.7	6.1	Aldehyde dehydrogenase, mitochondrial (ALDH CLASS 2) (ALDH1)	NC	338	448	0.4	524	0.5	398	0.5
7314	2.39	34	36.2	6.3	aldehyde reductase 1 (low Km aldose reductase)	NC	6982	6926	0.9	7067	0.9	6611	0.3
8308	2.43	28	39.6	6.8	aldolase C (AA 1-362)	NC	1326	990	0.6	2378	0.3	1742	0.5
806	1.39	10	184.6	4.9	alpha 1 type V collagen	NC	5738	4686	0.1	5899	0.8	5623	0.9
1618	2.43	42	73.2	5.5	aminopeptidase B	NC	152	236	0.3	193	0.5	183	0.5
8708	2.27	9	99.6	6.3	androgen receptor	NC	357	550	0.6	609	0.5	541	0.7
9107	**2.15**	**43**	**24.8**	**7.0**	**androgen receptor-associated protein 24**	**+**	**2609**	2501	0.7	2366	0.6	**3640**	**0.01**
310	1.64	22	35.4	5.0	annexin V	NC	1777	1994	0.3	1831	0.8	1762	0.9
4603	2.00	9	68	5.6	apoptosis inhibitor 2	NC	12921	13577	0.8	8483	0.2	18068	0.1
203	1.71	45	34	5.3	arylamine N-acetyltransferase	NC	1839	1983	0.3	1978	0.4	1816	0.9
7416	2.43	27	46.7	6.7	aspartate transaminase (EC 2.6.1.1), cytosolic – rat	NC	330	240	0.5	199	0.2	275	0.61
615	**1.54**	**16**	**48.2**	**4.3**	**calreticulin**	**+**	**5077**	**5787**	**0.02**	**6003**	**0.08**	**5707**	**0.05**
6509	2.43	42	57.8	6.0	chaperonin containing TCP-1 beta subunit (*Mus musculus*)	NC	3157	3612	0.1	2852	0.7	3151	1.0
2504	2.43	38	50.1	4.8	class I beta-tubulin	NC	1216	1528	0.2	1099	0.6	930	0.4
2505	2.43	39	50.1	4.8	class I beta-tubulin	NC	2020	1635	0.4	1764	0.5	1716	0.4
6002	2.43	32	15.7	5.9	Cu-Zn superoxide dismutase	NC	6232	6160	0.8	6565	0.3	5871	0.3
1805	2.43	33	92.7	4.7	Endoplasmin (94 kDa glucose-regulated protein) (GRP94)	NC	4481	3840	0.5	3357	0.2	3969	0.6
7402	1.72	18	47.4	6.2	Enolase, 1 alpha	NC	5680	6084	0.5	5695	0.9	5462	0.5
4406	1.35	27	49.4	5.4	eukaryotic initiation factor 5 (eIF-5)	NC	1135	1243	0.3	1031	0.6	1128	1.0
3422	2.43	22	51.1	5.0	GDP-dissociation inhibitor 1	NC	169	162	0.9	184	0.7	159	0.8
5711	2.43	19	88.5	6.1	glucocorticoid receptor	NC	133	160	0.7	137	0.9	147	0.9
3507	1.65	12	52.6	5.1	glucokinase	NC	135	136	0.9	83	0.3	154	0.6
7101	1.31	27	25.9	6.1	glutathione S-transferase Yb4 gene	NC	4724	4464	0.2	4337	0.3	3715	0.3
2806	2.43	16	91.2	5.1	GRIP-associated protein 1 short form	NC	613	459	0.4	520	0.6	367	0.3
2506	2.43	43	50.8	4.9	H^+-transporting ATP synthase; beta chain, mitochondrial	NC	3507	3753	0.5	3559	0.8	3503	1.0

SSP	z-Score	%	M_r	pI	Name	Change	Control	250 mg/m³	p	500 mg/m³	p	1000 mg/m³	p
5001	2.43	56	14.8	5.9	heart fatty acid binding protein	NC	4956	5034	0.8	5604	0.2	5314	0.2
3717	2.43	43	71.1	5.4	heat shock 70 kDa protein 8; Heat shock cognate protein 70	NC	4532	5719	0.2	4038	0.7	5072	0.5
3505	**2.43**	**19**	**57.8**	**4.8**	**heat shock factor 2**	+	**741**	**936**	**0.01**	852	0.3	895	0.2
3704	**2.43**	**24**	**85.2**	**4.9**	**heat shock protein 86**	−/+	**2278**	1034	0.3	**2987**	**0.05**	2657	0.7
2711	**2.07**	**29**	**85.2**	**4.9**	**heat shock protein 86**	+	**3103**	4460	0.2	4794	0.2	**5342**	**0.05**
9201	1.56	21	29.2	6.5	hydroxyacyl glutathione hydrolase; round spermatid protein RSP29	NC	2957	2839	0.7	3030	0.9	3421	0.2
9203	2.28	32	30.9	7.8	inducible carbonyl reductase	NC	1198	1241	0.8	1509	0.21	1535	0.2
3722	**1.39**	**15**	**71.7**	**5.4**	**integrin beta-7 subunit**	+	**279**	**604**	**0.02**	**632**	**0.01**	472	0.2
107	**2.11**	**28**	**22.5**	**4.9**	**interleukin-18**	+	**467**	**556**	**0.04**	**595**	**0.007**	496	0.5
3816	2.15	4	145.4	5.3	JNK/SAPK-associated protein-1 (*Mus musculus*)	NC	238	209	0.7	434	0.4	337	0.4
4218	**1.90**	**45**	**36.9**	**5.7**	**lactate dehydrogenase B**	+	**4467**	**5298**	**0.03**	4660	0.9	5033	0.4
3609	**2.43**	**17**	**66.8**	**5.2**	**lamin B1**	+	**574**	**816**	**0.002**	597	0.8	846	0.2
1110	2.43	57	27.9	4.7	mitochondrial import stimulation factor S1 chain	NC	466	955	0.3	838	0.3	602	0.6
4303	2.43	21	39.6	5.3	NDRG1 related protein NDRG2a2	NC	481	546	0.7	474	1	530	0.5
8609	**2.43**	**14**	**71.3**	**6.7**	**nicotinic acetylcholine receptor alpha 4 subunit**	+	**264**	658	0.4	971	0.1	**2020**	**0.001**
4409	2.43	40	49.6	5.4	nuclear RNA helicase, DECD variant of DEAD box family	NC	315	318	1	406	0.2	323	0.9
4819	2.43	37	95.2	5.5	Osmotic stress protein 94 (Heat shock 70-related protein APG-1)	NC	2908	3001	0.7	2765	0.6	3217	0.3
4822	**2.43**	**33**	**95.2**	**5.5**	**Osmotic stress protein 94 (Heat shock 70-related protein APG-1)**	+	**2403**	**2767**	**0.06**	2501	0.7	2680	0.4
3406	1.40	27	53.6	5.4	peripherin	NC	1133	1353	0.2	1324	0.3	1290	0.3
5103	2.43	44	24.9	6.0	peroxiredoxin 5; anti-oxidant protein 2	NC	492	500	0.9	692	0.4	654	0.6
3104	2.28	48	20.9	5.5	phosphatidylethanolamine binding protein	NC	13526	14393	0.5	14026	0.5	13957	0.6
3103	1.64	26	19.9	5.1	proline-rich protein	NC	3893	5581	0.3	5464	0.3	5433	0.3
5415	2.43	44	49.8	5.0	proteasome (prosome, macropain) 26S subunit, ATPase 3	NC	565	797	0.2	559	0.9	776	0.1
8209	1.67	17	23.1	7.0	proteasome (prosome, macropain) subunit, beta type, 2	NC	1199	1410	0.5	1170	0.9	1480	0.4
6501	2.43	26	57.1	5.9	Protein disulfide isomerase (Disulfide isomerase ER-60) (ERp60)	NC	2008	3674	0.3	1664	0.8	3344	0.4
2411	1.32	26	47.6	4.9	Protein disulfide isomerase (Protein disulfide isomerase P5)	NC	1651	1549	0.7	1672	0.9	1983	0.1
3601	2.43	23	73.1	5.0	protein disulfide isomerase related protein	NC	355	1534	0.2	859	0.3	872	0.3
509	**1.88**	**19**	**45.5**	**4.3**	**protein kinase C-binding protein Zeta1**	+	**171**	**256**	**0.01**	**255**	**0.002**	155	0.8
4307	2.43	37	35.9	5.1	Recombinant Rat Annexin V, Triple Mutant (T72k, S144k, S228k)	NC	1739	1776	0.8	1418	0.3	1688	0.8
7006	2.43	50	14.8	6.8	ribosomal protein S12, cytosolic	NC	3678	3762	0.8	3084	0.3	3045	0.3
4609	1.43	29	59.4	5.4	RP58 protein	NC	2398	2790	0.2	2178	0.5	2544	0.7
8312	2.43	31	38.8	7.9	serine dehydratase	NC	5737	5970	0.8	6236	0.7	7425	0.3

dehydrogenase (LDH) to the major metabolite methoxyacetic acid (MAA). EGME or MAA have been widely implicated in male reproductive system toxicity, including disruption of spermatogenesis (Berndtson & Foote, 1997). While the relationship between exposure to DiEGME and expression of ALDH has not been explored, Hobson et al. (1986) have indicated that dermal exposure (1 g/kg/d) of

guinea pigs to DiEGME 5 d/wk for 13 wk resulted in increased activity of LDH, whose abundance in the present study was increased ($P<.03$) 16% by the 250 mg/m^3 exposure. In view of the temperature sensitivity of spermatogenesis, it is not surprising that the major stress proteins (hsp and grp) are commonly expressed in the testis of rodent. These include 70 kDa hsp-1, hsp-2, and hsp-3; 70 kDa testis-specific hsp (hsp70t); 70 kDa heat shock cognate protein (hsc70); 75 kDa glucose-regulated protein (grp75); 78 kDa glucose-regulated protein (grp78); and heat shock 70-related APG-1 (osmotic stress protein 94) as well as regulators of the stress response such as heat shock factors 1 and 2. Repeated exposure of male rats to JP-8 resulted in differential expression of several proteins related to this stress protein family. Increased expression of heat shock factor 2 (low dose), total hsp70t (e.g., sum of SSP 622, 2604, 4603, 4608, 4611 charge isoforms) (high dose), Grp78 (low dose), and Hsp86 (moderate and high doses) suggests a generalized cellular stress response at those individual exposure levels. In addition, T-complex polypeptide 1 (TCP-1, aka chaperonin containing TCP-1 (CCT)) expression rose at high-dose exposure. TCP-1 is classified as one of the Type II molecular chaperones, the variety that has a specific role in the folding of newly synthesized tubulins and actins (Llorca et al., 1999) and in the mouse is encoded by testis-specific gene (Cctz-2) (Kubota et al., 1997) identified here. Previously, in in vitro test systems, TCP-1 levels were upregulated under continuous chemical stress with sodium arsenite as well as during recovery from chemical stress caused by sodium arsenite or a proline analogue, L-azetidine-2-carboxylic acid (Yokota et al., 2000). These results suggest that the stress proteins and chaperones mentioned above all may play a role in the recovery of cells from possible protein damage inflicted by JP-8, assisting in the folding of proteins that are actively synthesized and/or renatured as a consequence of prolonged exposure to the volatile components of JP-8. The slight (22%) but significant increase in Tat binding protein-1 (TBP-1) may be related to the response described above. TBP-1 is abundant in the testis and shows particular expression in spermatogonia, spermatocytes, spermatids and epididymal sperm. TBP-1 function is linked to the 26S proteasome and appears to be involved in numerous germ cell activities including ATP-dependent degradation of ubiquinized proteins (Rivkin et al., 1997). Surprisingly, the abundance of another chaperone, the endoplasmic reticular 150-kDa oxygen-regulated protein (ORP150), was decreased, particularly at the moderate JP-8 exposure. Though the mechanism is unclear, in neurons ORP150 is involved in cytosolic free calcium regulation, is induced by hypoxia/ischemia, and is absent in normoxia. The reason for ORP150's decreased expression in response to JP-8 is likewise unclear.

The interleukin-1 system is present in testis and is speculated to be involved in autocrine, paracrine and endocrine regulation of testicular cells and indirectly the process of spermatogenesis. However, interleukin-18 (identified as SSP 107) is associated with responses in tissue injury and induces apoptosis (Stoica et al., 2001) in response to JP-8 exposure. Although an increase in apoptosis was not observed in the present study, it should be noted that both interleukin-18 and lamin B (SSP 3609) are associated with the induction of apoptosis. This raises the possibility that JP-8 exposure, which has already been shown to induce apoptosis in the lung (Boulares et al., 2002), may also have induced the beginning stages of apoptosis in the exposed testis. Any direct effect of interleukins on Leydig cell function following JP-8 exposure was not evident, as there did not appear to be morphological changes in the Leydig cell population [unpublished observation]. Lamin B is found along the nuclear envelop of all cell types in the seminiferous tubule, including Sertoli cells, primitive type A spermatogonia, preleptotene, leptotene, zygotene and pachytene spermatocytes, and round spermatids (Moss et al., 1993).

Lamin B1 is a PKC binding protein, but nothing is known regarding this activity in the testis. However, it is noteworthy that JP-8 caused changes in the expression of another PKC binding protein in the testis, Zeta1 (SSP 509), a 50% increase a the low and moderate exposure. PKC binding proteins are abundant in the testis starting with the onset of spermiogenesis with high expression of specific mRNA in the elongating spermatids (Erlichman et al., 1999). Changes in these proteins following JP-8 exposure raise the possibility that subtle effects were taking place in the Sertoli cell cytoplasm or in the developing germ cells both of which depend on stable microtubule formations for normal spermatogenesis. Another upregulated (50%) cytoskeleton-related protein, A-kinase anchor protein (AKAP) (SSP2909), is expressed in high levels in the testis and is closely associated with the microtubule elements of the sperm

flagellum. Interestingly, it has also been suggested that AKAP 220 may play a role in targeting type II PKA for cAMP-responsive peroxisomal events (Lester et al., 1996). The nature of its altered expression here is elusive, but certainly warrants further investigation of potential long-term effects of JP-8 on spermatozoa formation and maturation.

The nearly 8-fold increase in alpha 4 subunit of the nicotinic acetylcholine (Ach) receptor in the high-doe JP-8 exposure group is difficult to explain. The testis does not appear to contain cholinergic nerve fibers, but does contain nACh receptors. These receptors can be found in blood vessels, mature germ cells and flagella of the sperm but not in the interstitial cells. Though nicotine has been shown to exert a negative effect on testicular function (Kavitharaj & Vijayammal, 1999), and alpha nACh is upregulated by interferon-gamma in the thymus (Zheng et al., 1999), the underlying reason for an apparent JP-8 related upregulation of the nACh subunit in the testis remains unclear.

Integrins are ubiquitously expressed on the surface of cells and enable cell-to-cell communication. The identification of SSP 3722 as the $\beta 7$ integrin is somewhat surprising as this integrin type is localized in the lymphocyte where it is involved in homing. Numerous subtypes are found in the testis, but integrin $\beta 7$ has not been described previously. A low abundance protein present in control testis, it's increased abundance at low and moderate JP-8 exposures may be related to an injury-related increase in the local lymphocyte population. However, it is also possible that, despite a reasonably high Z-score of 1.39 (92%), $\beta 7$ may have been misidentified and is actually one of the resident integrins. A potential explanation for changes in integrin expression is found in one of the more subtle observations that the lumen of certain seminiferous tubules was dilated after JP-8 exposure. It is well established that integrins are involved in mechanical stress to cells and tissues and thus, stretching of the seminiferous epithelium may have altered the expression of the integrin family of genes in the testis (Aikawa et al., 2002).

The effects of JP-8 on the male reproductive system described above are subtle. We are in the process of analyzing the testis in more detail but we currently have preliminary data suggesting that JP-8 may have induced an effect not only in the testis (proteomic response), but also an effect on the excurrent ducts of the testis. This effect would be related to fluid reabsorption in the head of the epididymis, as evidenced by the accumulation of fluid in the rete testis and seminiferous tubule lumens [Hess, unpublished results]. The basic pathophysiological mechanism involves the inhibition of fluid reabsorption by the efferent ductule epithelium. This epithelium is responsible for reabsorbing nearly 96% of the luminal fluids arriving from the rete testis, or the seminiferous tubule effluent. Disruption of this physiology results in either the accumulation of fluid or total occlusion, both of which creates back pressure on the rete testis and seminiferous tubules, which ultimately will lead to atrophy of the testis (Hess, 2002). Occlusion of the efferent ductules is not likely to have occurred in this study because testicular swelling or even doubling of testicular weight would have been a rapid response, which was not observed. Therefore, the most likely event was an inhibition of fluid reabsorption with subsequent accumulation of luminal fluid, which would explain the dilation of rete testis and seminiferous tubules. This is supported by the observation that serum albumin (SSP 6615) was increased (nearly two-fold) in the high-dose JP-8 treated testis. Such a response could indicate that fluid transport or even blood flow was disrupted, particularly in view of the fact that serum albumin is abundant in the male reproductive tract.

The observation that repeated low-dose JP-8 vapor exposure resulted in far more proteins with increased abundance than did exposure to the highest dose, and that exposure to the highest dose level resulted in significantly reduced abundance of twice as many proteins as the low dose exposure is difficult to explain but not unusual. This phenomenon has been observed consistently with JP-8 vapor and aerosol exposures in other organs [unpublished results] and its explanation requires additional study. Whether the alterations are related to injury versus adaptive or repair mechanisms, or perhaps to a more complex hormetic (Calabrese & Baldwin, 2003) response, remains to be determined.

3.4 Conclusion

Previously published studies have reported male reproductive system toxicity in humans or animals

exposed to hydrocarbon solvents. JP-8 jet fuel is a complex mixture containing over 228 hydrocarbon constituents, 3 performance additives, and at least four of the solvents previously identified as toxic to the male reproductive system. We used a 2D gel-based proteomic approach to investigate the potential reproductive toxicity in male rats of 91-day (6 hr/d) exposure to JP-8 in vapor phase. The goal of this animal research was identification of changes in protein abundance that may, in humans, predict subtle acute changes, or more complex chronic changes in reproductive system integrity or function. In the rat testis, it was clearly demonstrated that JP-8 vapor exposure, mimicking human occupational exposure scenarios, is sufficient to induce significant changes in the abundance of a number of different proteins with diverse or, in some cases, unknown functional significance. In the majority of cases, significant changes in the abundance of a specific protein occurred to one or two of the three JP-8 exposures concentrations, and in many cases there was a non-linear dose response. A simple explanation for this observation is that JP-8 vapor is itself a complex mixture that varies in content as a function of the concentration generated. It has been demonstrated, for example, that significant antagonism in toxic effects on the reproductive system can occur among hydrocarbon solvents during certain concurrent exposures. This suggests that even subtle changes in the formulation of widely used jet fuels, including changes in performance additive packages (e.g., ongoing conversion from JP-8 to the JP-8+100 formulation) and batch variations among and within jet fuel manufacturing operations (i.e., manufacturing to meet fuel performance and not fuel content requirements), may require toxicological evaluation prior to widespread implementation.

While there is minimal evidence of reproductive toxicity in humans exposed occupationally to JP-8, there have, however, been no published efforts to scientifically evaluate this possibility. Thus, the protein abundance modulations observed in the present 91-day animal study are somewhat disquieting, raising the possibility that, by extrapolation, JP-8 may have significant effects in humans, especially when exposed to the fuel during careers that may exceed 30 years. Admittedly, the approach taken here is limited in its sensitivity to very low abundance proteins, and the nature and functional significance of identified changes in protein expression remains to be fully explained by further research, such as field testing of career fuel workers for subtle changes in reproductive system function.

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